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13. ABSTRACT *(Maximum 200*

Progress through the cell cycle requires a concerted interplay between the growth promoting cyclin-dependent kinases (CDKs) and inhibitors such as p21/WAF1 that suppress the CDKs (CKIs). Despite the apparent ability of p21 to inhibit CDKs in vitro, its ability to inhibit these kinases in vivo has been a matter of controversy. In addition, while it is known that p21 participates in several complexes with the DNA polymerase processivity factor, PCNA, and cyclin and CDK proteins, it is not clear whether the full complement of p21-associated proteins has been defined. Nor has the function of such multimeric complexes in normal and transformed cells been thoroughly characterized. In light of experiments that indicate an important role for p21 as a downstream target of the p53 checkpoint protein and the observed defective response to DNA damage in mice lacking the p21 gene, it is essential that we understand the role of this protein in normal cells and how perturbations in its expression levels and/or function can lead to de-regulated growth. We are therefore attempting to understand the biochemical function of p21 and associated complexes to determine whether de-regulation of complex formation can be associated with transformation of human mammary cells.

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Brian Dynlacht 8/5/99

PI - Signature Date

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5. INTRODUCTION

Background

The cyclin-dependent kinases (or CDKs) are an extended family of mammalian serine/threonine protein kinases containing at least nine members (CDC2 and CDKs 2-9) related by several structural features. Regulation of CDK activity occurs at several levels. First, activation of each requires binding to a distinct cyclin regulatory subunit. Thus, CDC2 pairs specifically with cyclins A and B, while CDK2 combines with cyclins A and E. The D type cyclins pair exclusively with CDK4 and CDK6. Second, activation of most CDK subunits requires phosphorylation of a highly conserved threonine residue. In addition to phosphorylation, activation of CDKs requires de-phosphorylation of inhibitory residues by the cell-cycle regulated phosphatase, Cdc25.

Cyclin complexes with CDKs 2, 4, and 6 are thought to be required for progression through the G1 phase of the cell cycle and entrance into S phase. CDC2 activity is required later in the cell cycle for the G2-to-M phase transition. These kinases are thought to function by phosphorylating, and thus modifying the activity of, important downstream targets. Although several substrates of CDKs have been identified through in vitro assays, comparatively little is known about the identity of significant physiological substrates regulated by the CDK family during progression into S phase. CDK2 is known to phosphorylate the retinoblastoma tumor suppressor protein (pRB), and the transcription factor E2F known to play a pivotal role in cell growth by virtue of its ability to activate genes necessary for S phase entry (reviewed in Dyson, 1998). E2F and pRB are thought to be physiologically important substrates for the CDK2 kinase, since their activity is modulated by this kinase, and both proteins are phosphorylated in vivo on sites that overlap with those modified in vitro (reviewed in Weinberg, 1995). Phosphorylation of either pRB or E2F inhibits the activity of these proteins.

The growth-promoting activity of CDKs is opposed by another class of regulators, the CDK inhibitors (CKIs) that suppress growth. At least seven distinct CKIs have been identified, and they fall into two sub-groups. The INK4 inhibitors of CDK4 include the p15, p16, p18, and p19 proteins that bind to, and inhbit, the cyclin D-associated kinases, CDK4 and CDK6. A second class of CKIs consists of p21 (known also as WAF1/Cip1/Sdi1/CAP20), p27Kip1, and p57Kip2, each of which is capable of inhibiting in vitro each of the CDKs involved in cell cycle progression. The importance of several of these CKIs has been established through mouse knock-out experiments which indicate that these proteins play an important inhibitory role in cell proliferation (reviewed in Sherr and Roberts, 1999). Furthermore, one CKI, p21, is a known downstream target of the p53 protein essential for the DNA damage checkpoint. p21 levels rise in response to DNA damage as a result of p53 activation, and this checkpoint is known to be defective in most cancers. It is thought that increased amounts of p21 sequester cyclin/CDK complexes, inhibiting their activity, and preventing transit into S phase.

Given this central role for the p21 family of inhibitors in restraining the cell cycle, we have begun to investigate the composition and function of complexes that contain p21, the related inhibitors p27Kip1 and p57Kip2, and the pRB-related proteins p107 and p130 that also appear to bind E2F as well as inhibit associated cyclin/CDK complexes. Our previous studies suggested that normal cells contain the full complement of cyclins (A, B, D, and E) and CDKs (CDC2, CDK2, CDK4, CDK6) as well as PCNA, but that several transformed cell lines and at least one breast cancer cell line lacked certain p21 complexes. We have also found that in normal cells, p21 forms a complex with E2F-4, but the function of this complex is not yet known. Our studies described below are an attempt to further understand the assembly and function of p21 complexes in normal mammary cells and compare them with analogous complexes in breast cancer cells.

6. BODY

1) p21 complexes in normal and cancerous mammary cells

A) Comparison of levels of p21 and associated proteins in normal, primary mammary cells, their matched cancerous derivatives, and established mammary cell lines
B) Correlation between existence of 'free' p21 and growth control
C) Identification of novel polypeptides immunoprecipitated specifically by anti-p21 monoclonal antibodies

A) Comparison of levels of p21 and associated proteins in normal, primary mammary cells, their matched cancerous derivatives, and established mammary cell lines

Growth of matched, primary normal mammary and breast cancer cells
Growth of such matched cell lines presented a number of advantages but also one disadvantage, namely, that these cell lines had not been previously characterized by ATCC. In addition, growth of primary, normal mammary cells (Hs578Bst, Hs606, Hs608) obtained from ATCC presented us initially with a significant impediment. Among these cells, two of them (Hs578Bst and Hs606) posed the greatest challenge. These cells were refractory to growth in standard media (DMEM containing 10% Gibco fetal bovine serum), even at low passage number, leading to doubling times greater than three days and eventually cell senescence. After testing several types of serum and supplements, we eventually found media that enabled growth (albeit much reduced relative to other normal and transformed mammary cell lines). This media contained 20% fetal bovine serum (from Hyclone), and some cells (such as Hs578Bst) also required 30 ng/ml epidermal growth factor (EGF) and an insulin/transferrin/selenium mixture. Even with these media supplements, the primary cells grew very slowly, taking several months to accumulate cells for freezing stocks and performing experiments. All of the following comparisons between normal and cancer cells were made with cells at low passage number grown with identical supplements.

We began characterizing each of these cells by performing western blotting of whole cell extracts and probing with all known components of p21 complexes. A summary of these data is given in Table 1 as levels of protein compared with normal human WI38 cells or by comparing matched cells.

Table 1
Protein (detected by direct western blotting)

cell line	p21	PCNA	cycA	cdk2	cycD	cycE
VA13	reduced	elevated	elevated	equal	~equal	reduced
T47D	reduced	elevated	elevated	equal	reduced	reduced
MCF7	similar	elevated	elevated	equal	equal	equal
MDA-MB-468	reduced	elevated	elevated	equal	reduced	
Hs578Bst	reduced	reduced	equal	equal	equal	equal
Hs578T	reduced	elevated	elevated	equal	equal	equal
ZR-75	equal	elevated	elevated	equal	equal	equal

cells	p21	PCNA	cycA	cycE
Hs 578T	reduced	elevated	elevated	elevated
Hs 606	reduced	elevated	elevated	elevated
Hs 608	~equal	~equal	~equal	~equal

Table 1. Comparison of p21 associated proteins in cell lines under study. Cell lines in the upper panel were compared on the basis of WI38 protein levels, while those in the lower panel were compared between matched sets, i.e., the breast tumor cells (Hs578T, Hs606T, and Hs608T) were compared with normal mammary cells (Hs578Bst, Hs606, and Hs608).

B) Correlation between existence of 'free' p21 and growth control

We had found previously (Cai and Dynlacht, 1998) that one of our anti-p21 monoclonal antibodies, CP55, was uniquely capable of immuno-purifying 'free' p21, that is, a form of p21 that lacked associated proteins. Using this antibody, we determined that normal WI38 fibroblasts had significant amounts of 'free' p21, while the SV40-transformed derivative of this cell line (WI38-VA13) had barely detectable amounts (see 1998 Annual Report). We had also observed that a breast cancer cell line, T47D, also lacked detectable amounts of 'free' p21. Interestingly, we also observed that a second antibody (CP36) that exclusively recognized cyclin A/CDK2 immunoprecipitated nearly undetectable amounts of p21 from T47D compared to an antibody (CP68) that immunoprecipitated all p21/cyclin/CDK/PCNA complexes. This suggested a profound de-regulation of certain p21 complexes in the two breast cancer cell lines tested.

We were concerned, however, that the results might reflect a diminution in overall levels of p21 in certain cell lines (in most cases 2-3-fold) relative to normal ones due to decreases in positive regulators, such as p53. To circumvent this complication, all further experiments were performed using normalized amounts of extract that yielded similar amounts of p21 in western blots of CP68 immunoprecipitates. We screened a number of normal cell lines (WI38, Hs578Bst) and cancer cell lines (WI38-VA13, MDA-MB-468, ZR-75, Hs578T, MCF-7, T47D, the latter five of which are mammary cancer cell lines). We were especially interested in comparing two sets of matched cell lines, WI38/WI38-VA13 and Hs578Bst/Hs578T. The latter pair was most interesting to us because it represented a matched set of early passage normal and cancer cells (from ATCC) from the same patient. Using this strategy, we obtained the following provocative and reproducible data.

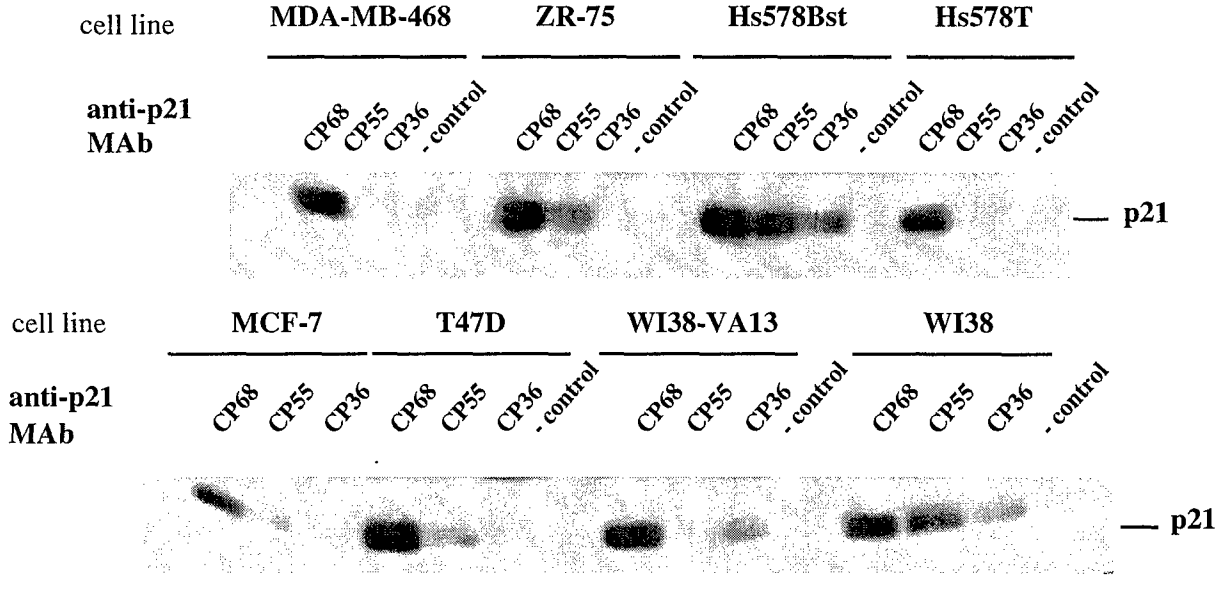

Figure 1. Loss of specific p21 complexes from breast cancer cell lines. 0.2-1 mg of protein from whole cell lysates of various cell lines was immunoprecipitated with the indicated anti-p21 or negative control antibodies and subjected to western blotting for the p21 protein.

If one compares the first immunoprecipitation of each set (CP68) with others (CP55 and CP36), one finds that in normal cells, approximately one-half the amount of p21 is present as 'free' p21 and about one-fifth is present in the cyclin A/CDK2 complex. Such a pattern was completely perturbed in each cancer cell tested. Thus, even longer exposures revealed the absence of p21 in CP36 IPs of MDA-MB-468, ZR-75, Hs578T, MCF-7 and T47D) and a significant diminution or loss of p21 in CP55 IPs of every cancer cell tested (with a complete loss in MDA, Hs578T, and WI38-VA13 cells). These experiments demonstrated that loss of specific p21 complexes was not due to decreased levels of p21 protein. Rather, there was a good correlation between loss of specific p21 complexes and loss of a normal growth phenotype.

These experiments were repeated several times, and the data are summarized in the following table (Table 2).

Table 2
Antibody/p21 amount in IP

Cell Line	CP36	CP55
VA13	equal	reduced (none)
T47D	reduced (none)	reduced
MCF7	reduced (none)	reduced
MDA-MB-468	reduced (none)	reduced (none)
Hs578Bst	equal	equal
Hs578T	reduced	reduced (none)
ZR-75	reduced	equal

Antibody/
p21 amount in IP

cell	CP36	CP55
Hs 578T	reduced	reduced
Hs 606T	equal	equal
Hs 608T	equal	equal

Table 2. Amounts of p21 immunoprecipitated from extracts of various mammary cell lines. Levels of p21 were compared with normal human WI38 cells (upper panel) or with extracts of normal matched mammary cells (lower panel).

Importantly, we further confirmed that this observation was not the result of decreased amounts of cyclin A or cyclin E associated with CDK2 by probing the above western blots with anti-cyclin antibodies and by performing parallel IPs with CDK2 and cyclin antibodies.

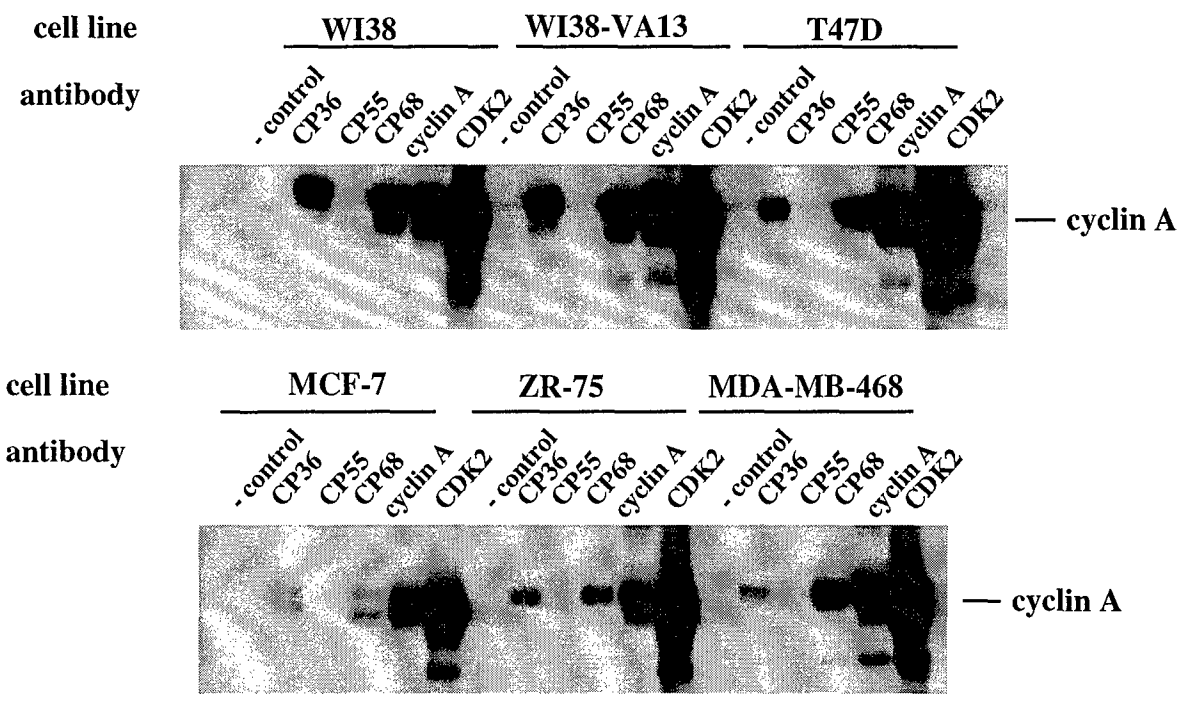

Figure 2. Decreased association of p21 with cyclin A in breast cancer cells. Anti-p21, cyclin A, and CDK2 immunoprecipitates were screened for co-precipitation of cyclin A.

The important conclusion from these studies was that despite equal or greater amounts of each target protein in the immunoprecipitations, the breast cancer cell lines showed definitive decreases in certain cyclin/CDK complexes associated with p21. Thus, although anti-cyclin A or CDK2 antibodies precipitated similar amounts of cyclin A protein from WI38, T47D, MCF-7, ZR-75, and MDA-MB-468, the amount of this protein associated with p21 was barely detectable in many of the cancer cell lines (hence the over-exposed blot in Figure 2). It is also important to note that most of the cell lines under study had equal or elevated levels of cyclin A and other cyclins in crude lysates (Table 1), ruling this out as contributing to decreased levels of cyclin association with p21.

Despite these intriguing data with several breast cancer cell lines and a set of matched normal primary cells and tumor cells, two other matched sets obtained from The Naval Biosciences Laboratory (NBL), Hs606 and Hs608, suggested equal amounts of cyclin A-associated and 'free' p21 in both CP36 and CP55 IPs.

It is possible that diminution of 'free' p21 in cancer cells is nevertheless a rather general phenomenon and that the Hs606 and Hs608 cells are atypical. Also, because ATCC does not warrantee these particular cells (they were obtained by ATCC from NBL in Oakland, CA, but have not been previously characterized by ATCC), another strong possibility is that the cells are a mixed population of normal and cancer cells. Indeed, the ATCC catalog provides this caveat. Because of the reproducible and definitive results obtained above with well characterized cancer cells obtained from ATCC that lack contamination with normal cells (Figure 1) and the fact that contrary data were obtained with cells that may be mixed populations, we have attempted to obtain additional matched pairs of normal and tumor cells from colleagues at Harvard Medical School. Although we have not succeeded in obtaining such cells, we will continue our efforts to obtain additional matched normal and cancerous mammary cells. We believe these are important experiments because the exact reason for the multitude of p21-cyclin/CDK complexes in normal cycling cells

has not been established nor has any strict correlation been made between loss of function of such complexes and tumorigenesis. Ideally, we would like to establish a link between loss of excess ('free') p21 or p21/cyclin A/CDK complexes and the transformed phenotype. Such links may have some value for breast cancer prognosis or therapeutics in the future.

C) Identification of novel polypeptides immunoprecipitated specifically by anti-p21 monoclonal antibodies

As described in our initial Statement of Work, we had identified through immunoprecipitation of metabolically labeled extracts a number of polypeptides that did not correspond to known p21-associated polypeptides. Thus, we had ruled out PCNA and all known cyclins and CDKs by parallel immunoprecipitations with antibodies directed against these proteins and gel electrophoresis. In multiple experiments (documented in a previous Annual Report), we demonstrated that these polypeptides were present in anti-p21 immunoprecipitates of normal human cells but not in several transformed cell lines. We therefore sought to develop a purification strategy to isolate sufficient quanitities of each of these putative novel proteins to obtain peptide sequence information.

Given our documented success in obtaining highly purified p21 complexes from metabolically labeled extracts using our panel of monoclonal antibodies, we attempted the immunopurification of such complexes from normal human fibroblasts (WI38) as well as two mammary cell lines expressing significant quantities of p21 (MCF7 and T47D).

Identification of the putative 28 kDa p21-associated protein

We therefore scaled up our purification strategy, beginning with several milligrams of protein from WI38 and MCF7 lysate. After immuno-purification, complexes were electrophoresed, gels were stained with a Colloidal Coomassie Blue, revealing a yield of approximately 100 ng of each protein. Because the position of p28 was very close to that of the antibody light chain, we attempted to release p28 from the coupled antibodies using chaotropic reagents. We found that treatment with RIPA buffer containing 0.5% deoxycholate quantitatively released p28 from the antibody-sepharose conjugate (Figure 3).

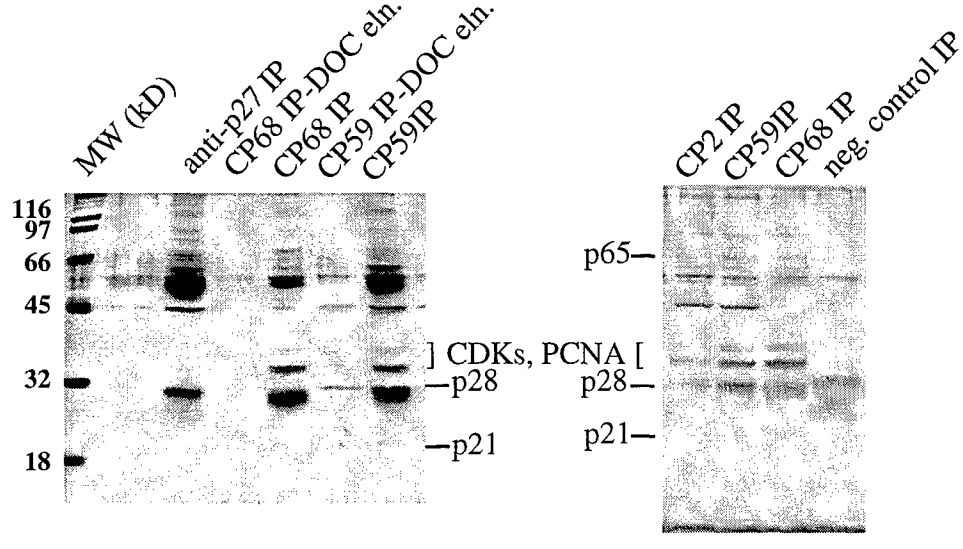

Figure 3. Immuno-purification of putative p21-associated proteins. Approximately 3-5 mg of protein from MCF-7 whole cell extracts were immuno-purified with the indicated

antibodies and the resulting SDS-PAGE gels were silver stained. Parallel IPs were performed using a negative control anti-hemagglutinin (HA) antibody. To resolve p28 from the antibody light chain, immunoprecipitates were eluted with deoxycholate (lanes labeled 'DOC eln.). Cyclin, CDK, PCNA, p21, as well as the previously unidentified p28 and p65 polypeptides (indicated) were reproducibly detected in anti-p21 but not control Ips. CDKs were weakly detected in anti-p27 immuoprecipitates. Immunopurification was scaled up, and the p28 and p65 bands were excised and subjected to proteolysis in situ and mass spectrometric sequencing.

The relevant bands were excised and were given to William Lane at the Harvard Microchemical Facility in our building. Proteins were trypsinized in situ, eluted, and subjected to mass spectrometry and sequence analysis. Peptides obtained were compared with a human protein sequence database, and both proteins were identified as described below.

Seven peptides (Figure 4) derived from a tryptic digest of p28 were sequenced.

GPSWDPFR
LFDQAFGLPR
LPEEWSQWLGGSSWPGYVRPLPPAAIESPAVAAPAYSR
QLSSGVSEIR
VSLDVNHFAPDELTVK
DGVVEITGK
YTLPPGVDPTQVSSSLSPEGTLTVEAPMPK

Figure 4. Peptides obtained from a tryptic digest of p28 excised from gels.

Each of these peptides was identical to ones found in the 27 kDa human heat shock protein (hsp27). We were quite intrigued by this result for a number of reasons. First, the expression of hsp27 in ovarian and breast cancer cell lines has been correlated with resistance to the chemotherapeutic drugs, cisplatin and doxorubicin, and hsp27 expression in a number of cancers was linked to poor prognosis (Arts et al., 1999). Second, several reports have documented the association between human cyclins and heat-shock/chaperonin proteins necessary for their folding and activity. For example, the chaperonin CCT was found to bind to human cyclin E, allowing it to fold properly and associate with CDK2 (Won et al., 1998). In addition, the p50Cdc37 protein was shown to act by targeting the association of the Hsp90 chaperonin to CDK4, stabilizing this CDK and enabling it to associate with cyclin D (Stepanova et al., 1996).

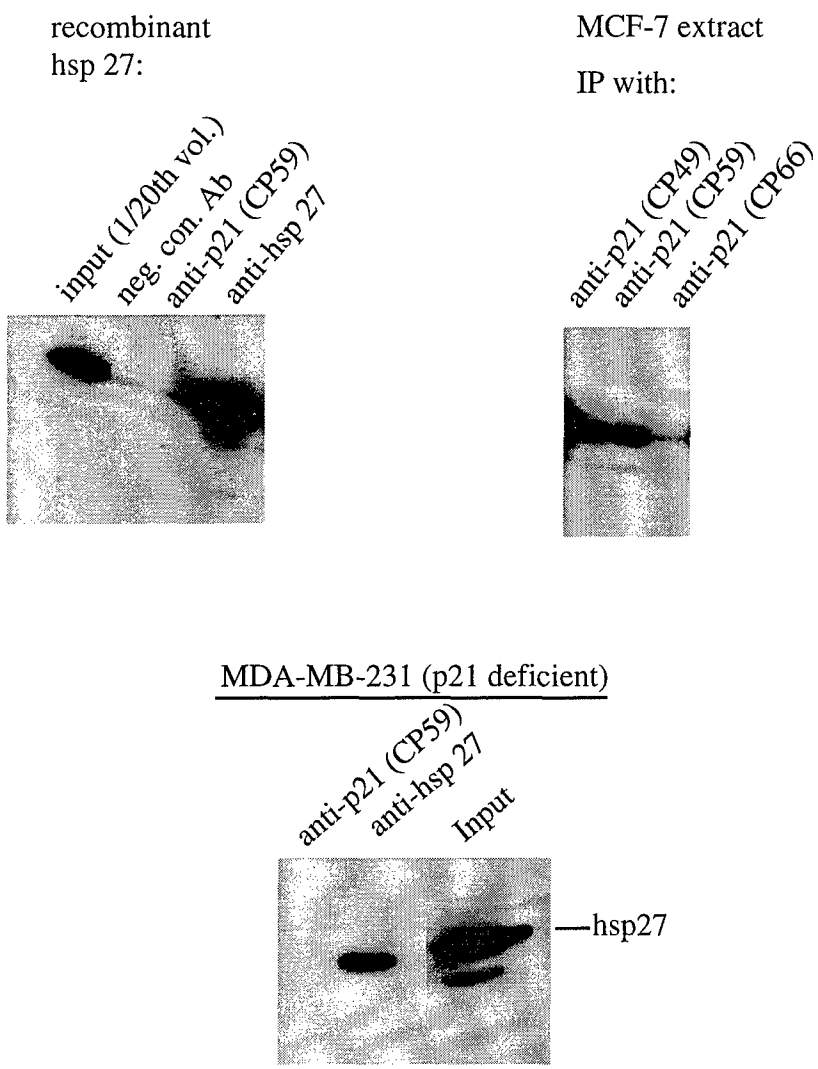

Figure 5. p28 is identical to hsp 27. In the upper left panel, recombinant hsp 27 was not immunoprecipitated by CP59 but was precipitated by anti-hsp27 antibodies, suggesting specificity. MCF-7 extracts, which contain p21 and hsp 27 were immunoprecipitated with several anti-p21 antibodies, and the resulting immune complexes were immunoblotted for hsp27. CP49 and 59 immunoprecipitated hsp27 from such extracts. The breast cancer cell line MDA-MB-231 has no detectable p21 but abundant amounts of hsp 27, which are precipitated by anti-hsp 27 antibodies but not by CP59.

We therefore obtained commercially available anti-hsp27 antibodies (Stressgen) and antibodies from our colleagues. We also obtained expression plasmids to overproduce the protein in mammalian cells and in an in vitro transcription/translation system. We were successful in immunoprecipitating hsp27 from WI38 and MCF7 cell extracts using either anti-hsp27 antibodies or specfic anti-p21 antibodies (CP49, CP59 both of which have overlapping epitopes; see previous Annual Report)(Figure 5). We have also examined extracts of other cell lines that apparently lack p21 (the breast cancer cell line MDA-MB-231) and have abundant amounts of hsp27, and these do not display precipitation of hsp27 protein with anti-p21 antibodies. The caveat here is that hsp27 was detected in anti-p21 immunoprecipitates of extracts from certain cell lines that contain very low

levels of p21 (such as 293 cells, in which p21 is barely detectable by western blotting). In addition, when we mixed recombinant hsp27 and mixed this protein with cell extracts or recombinant p21, both proteins were immunoprecipitated in anti-p21 immunoprecipitations (Figure 5). However, in some experiments, we also detected hsp27 in anti-p21 immunoprecipitates in the absence of recombinant p21 when low stringency buffer conditions were used (100 mM KCl, 0.1% NP-40). Co-immunoprecipitation was highly sensitive to experimental conditions, particularly salt and detergent concentrations, and hsp 27 was not immunoprecipitated in the absence of p21 when slightly more stringent conditions (250 mM NaCl) were used.

At present we do not know the basis of these contradictory results, and further work will be needed to assess the relevance of hsp27 association with p21 complexes. It has been established by several investigators that hsp27 multimerizes to form very large complexes, and we have noticed heterogeneity of hsp27 complexes in glycerol gradients (data not shown). The aggregation of hsp27 may be a source of non-specific precipitation. Therefore, more stringent wash conditions can be examined and additional cell lines that lack the p21 gene will be used for these experiments because they represent a more defined p21-/- background than the cell lines we have previously examined.

A putative p21-associated 65 kDa polypeptide

We had also identified a polypeptide in certain anti-p21 immunoprecipitates that was not purified using control antibodies. We therefore excised this band (Figure 3) and once again obtained greater than 10 peptides that were sequenced. This 65 kDa protein was identified as the translocation in liposarcoma (TLS or FUS) protein. The latter protein was of interest because translocation breakpoints in the TLS gene (which encodes an RNA binding protein of unknown function) have been implicated in several leukemias and sarcomas. A colleague, Dr. David Ron (Skirball Institute), was generous in providing us with high affinity anti-TLS antibodies. We therefore performed immunoprecipitations with several of our anti-p21 monoclonal antibodies and immunoblotted the resulting immunoprecipitates. We could demonstrate that p21 and TLS were indeed co-immunoprecipitated from WI38 and MCF 7 extracts. However, when we performed analogous experiments with cells lacking p21 (including MDA-MB-231, 293, and MOLT-4), we again co-immunoprecipitated the TLS protein (negative data not shown). We conclude that although the 65 kDa polypeptide obtained in our immunoprecipitates appeared to be specifically purified by several of our antibodies, the TLS protein was non-specifically purified by this protocol. This is consistent with the observations of others that TLS is a 'sticky' protein that associates with many proteins non-specifically (D. Ron, pers. comm.). Furthermore, we have expressed recombinant TLS by coupled in vitro transcription/translation and several antibodies immunoprecipitated TLS in the absence of added p21, again suggesting non-specific cross-reactions. In light of these results, we have decided not to pursue TLS as a candidate p21-associated protein.

Identification of putative p21-associated protein, p40

One of our anti-p21 antibodies, CP2, immunoprecipitated a polypeptide of approxiately 40 kDa preferentially from normal cells as cells proliferated. We were thus very interested in obtaining peptide sequence information for this protein as well. However, we have not succeeded in obtaining enough p40 polypeptide by immunopurification to perform mass spectrometric sequencing. During the course of our studies, we were contacted by a colleague studying a 39 kDa onco-protein thought to associate with p21. Preliminary data suggest that this protein may correspond to our putative novel p40 band identified in CP2 IPs, but further work will be necessary to confirm this.

Future work will therefore be directed toward confirming the identity of the 39 kDa onco-protein presumed to associate with p21 complexes. We will also continue to attempt to detect an association between hsp27 and p21 complexes in cell lines that are null for the p21 gene. To this end, we have obtained two types of p21-/- cells, including mouse embryo fibroblasts (MEFs; a

kind gift of T. Jacks) and HCT116 human colon carcinoma cells in which p21 has been deleted by homologous recombination (a kind gift of B. Vogelstein). For purposes of comparison, we will attempt to detect both the 39 kDa and hsp27 proteins in complexes that contain the related CKIs p27Kip1 and p57Kip2 to determine whether these proteins specifically interact with p21 or the entire family of p21 related inhibitors.

2) **Characterization of p21-E2F complexes**

We have previously shown that we could detect cellular complexes between p21 and the transcription factor E2F-4/DP-1 using a subset of our anti-p21 monoclonal antibodies. In addition, the interaction was specific because no E2F heterodimers were detected in immunoprecipitates of mouse embryo fibroblast (MEFs) extracts deficient in p21.

In order to dissect the interaction between E2F and p21, we decided to reconstitute this interaction in tissue culture cells by ectopic expression. We reasoned that his would allow us to (1) examine each of the proteins and domains required for this interaction, as well as interaction specificity and (2) test the functional consequences of such an interaction.

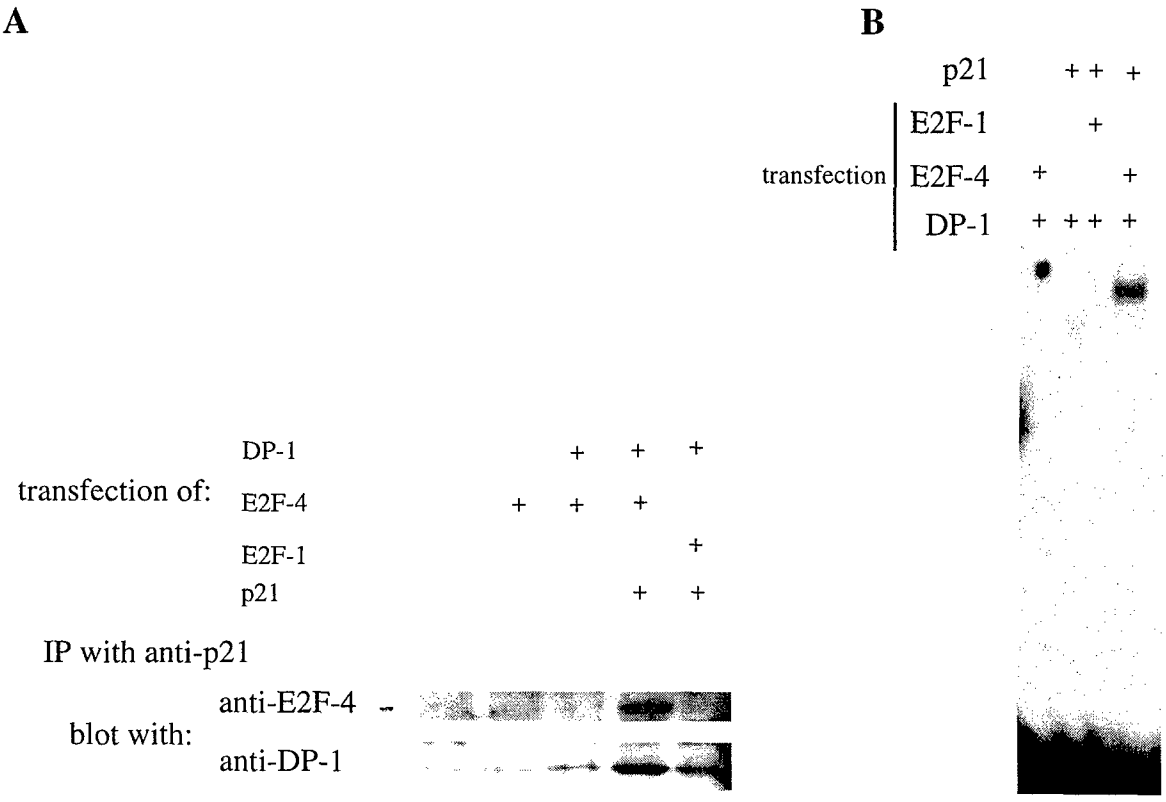

Figure 6. Expression of p21 proteins, E2Fs, and DP-1 by transient transfection and reconstitution of complex formation in vivo. 293 cells (A) or C33A (B) cells, both of which lack p21, were transfected and extracts were immunoprecipitated with anti-p21 antibodies (CP68). The resulting immunoprecipitates were probed (A) on western blots with antibodies against E2F-4 and DP-1 or (B) deoxycholate released and subjected to gel mobility shift analysis. Complex formation requires the presence of E2F-4, DP-1, and p21.

These experiments suggested the following. First, and most importantly, we have recapitulated the specificity of interaction between E2F family members and p21, since E2F-4 was uniquely capable of interacting with p21, while no other E2Fs were so able (Figure 6 and data not shown). Secondly, we could show that the interaction required both E2F-4 and DP-1, since expression of either protein alone failed to produce a complex containing all three proteins. The ternary complex is still active for DNA-binding since the E2F-4/DP-1 complex released from an anti-p21 immunoprecipitate had significant DNA-binding activity (Figure 6B). However, complex formation between E2F-1/DP-1 and p21 did not require DNA, since complex formation was not affected by incubation of lysates with ethidium bromide prior to immunoprecipitation (data not shown).

Success in this assay allows us to determine the domain(s) of p21 involved in the interaction with E2F-4/DP-1. To this end, we also expressed an N-terminal deletion mutant of p21 that includes the cyclin and CDK binding domains. Interestingly, this domain alone was unable to interact with the combination of co-expressed E2F-4 and DP-1 (Figure 7). This suggests that the carboxy-terminus of p21 is required for the interaction with E2F. We have also attempted to repeat this experiment with a carboxy-terminal deletion of p21. Unfortunately, we have not had success in expressing this deletion mutant.

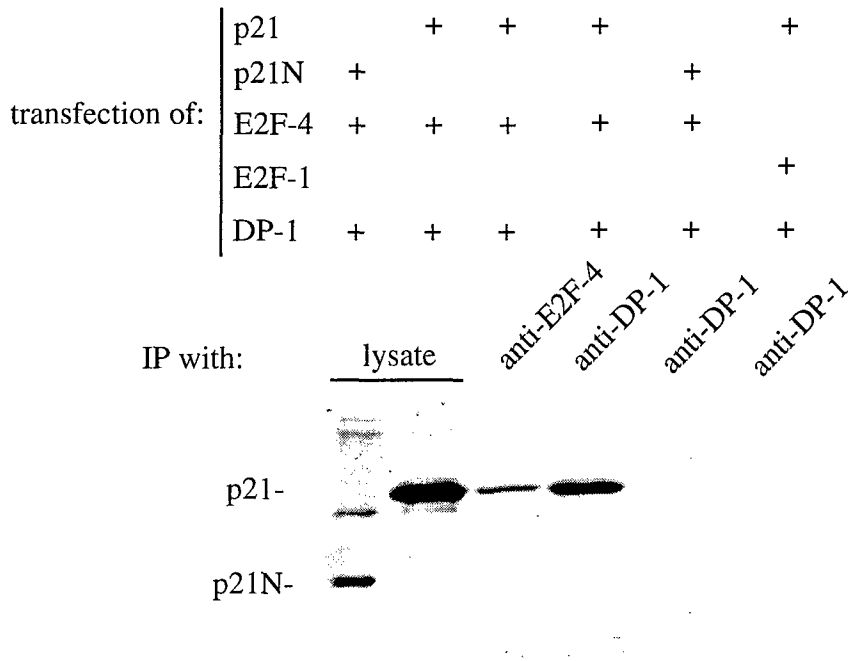

Figure 7. The amino-terminus of p21 is unable to associate with E2F complexes in vivo. 293 cells were transfected with the indicated constructs and immunoprecipitated with antibodies against E2F-4 or DP-1 as shown, and western blots of these immunoprecipitates were probed with anti-p21 antibodies.

Our future efforts will therefore be directed toward trying to express this carboxy-terminal truncation mutant by sub-cloning the cDNA insert into another expression vector to overcome low levels of protein production. We have also generated expression vectors to produce a number of small internal deletions of p21 that remove the cyclin binding domain, the CDK binding domain, or both and, in parallel, p57 deletion mutants. Once produced, we will test whether these mutant proteins can associate with E2F in vivo by transient transfection. We can also simultaneously examine the association of endogenous and transfected cyclins and CDKs with p21/E2F complexes

to determine which complexes can form in vivo. We will then test the effect of each on E2F transcription activity using chloramphenicol acetyltransferase (CAT) reporter assays. Full-length and truncated forms of p21 (ones that do and do not interact with E2F) can be tested in parallel.

We have also very recently devised a chromatin immunoprecipitation (ChIP) procedure to examine the possible association of p21 with endogenous genes. Currently, this protocol works well for immunoprecipitation of most E2F family members, and using antibodies against these proteins as positive controls, we will attempt to immunoprecipitate p21 and associated chromatin using our panel of anti-p21 antibodies. We will first attempt to study whether p21 associates with known E2F responsive genes by amplifying immunoprecipitated chromatin with several pairs of primers that hybridize to previously characterized genes (including, for example, E2F-1, cyclin E, B-Myb, p107). If this approach does not work, it may be necessary to clone the DNA sequences obtained from anti-p21 immunoprecipitates to identify novel genes that associate with the p21/E2F complex.

7. Key Research Accomplishments

--Characterized loss of specific p21-containing complexes ('free' p21 and cyclin A/CDK2/p21) in breast cancer cells relative to normal cells

--Identification of putative p21-associated protein as Hsp 27.

--Reconstitution of the p21-E2F4 interaction by transient transfection and preliminary characterization of proteins and domains necessary and sufficient for the interaction.

8. Reportable Outcomes

Presentations:
Presentations at Harvard University Departmental Retreat (September 1998) and to a Boston-based cell cycle group (October 1998)

Presentation of this work and publication (Cai et al., 1998) have resulted in our distribution of each of the anti-p21 monoclonal antibodies characterized through this grant to many colleagues in the field of cell cycle and cancer biology.

Funding applied for based on earlier work supported by this grant:
1999 Breast Cancer Research IDEA Award. The IDEA Award grant does not overlap scientifically or financially with this current grant.

Employment opportunities applied for/obtained:
A postdoctoral Fellow supported by this Grant, Kang Cai, has obtained a position in Bayer Corporation, Chapel Hill, NC

9. Conclusions

Our findings using breast cancer cell lines and one pair of matched normal and tumor cells suggest the interesting possibility that a hallmark of certain breast cancer lines is the loss of specific p21 complexes and not others. Specifically, 'free' p21 that does not reside in cyclin/CDK complexes and cyclin A/CDK2/p21 complexes were strikingly diminished or absent. This conclusion is based on the observation that expression levels of cyclins and CDKs themselves do not decrease in these cells; indeed our experiments (Table 1) and other published work suggests these proteins may be

elevated in cancer cells. In addition, the use of normalized extracts circumvented the problem of slightly reduced p21 levels in cancer cells. However, two other matched cell pairs obtained from NBL suggested otherwise, namely, that these complexes were not altered in breast cancer cell lines. However, we believe, based on caveats in the ATCC/NBL catalog, that contamination with normal cells may obscure our results, and we must continue to test this idea using other specimens, if possible, that lack such contamination. Ultimately, these experiments may be important to our understanding of the biological function of each of the distinct p21 containing complexes.

We have made progress in identifying putative uncharacterized p21-associated proteins. We have identified the p28 polypeptide immunoprecipitated by two monoclonal antibodies (CP49, CP59) as the Hsp 27 protein. Although certain experiments suggest an association between this protein and p21, others suggest that this is not the case. Because Hsp 27 is notorious for its ability to form very large homomeric complexes of several hundred kDa, we must be careful to rule out non-specific effects. We will therefore attempt to isolate complexes containing both proteins from cells that are deficient for the p21 gene. This may allow us to definitively rule out any non-specific recognition of Hsp 27 by our anti-p21 antibodies. These experiments could be of future significance given the role of chaperonins in assembling cyclin/CDK complexes and the correlation between Hsp 27 levels and disease prognosis.

We have also made progress in reconstituting the p21-E2F complex in cells in order to examine the factors and domains required for assembly of the complex. We have found that assembly requires both E2F-4, DP-1, and p21, and that association does not require the CDK binding domain of p21 and may require a domain(s) of p21 in addition to its amino-terminus. We are currently investigating the regions of p21 necessary for association and the potential association of cyclins and CDKs to determine the overall architecture of p21-E2F complexes. Our goal is to eventually identify all components of the p21-E2F complex and to determine whether it associates with chromatin. If so, it will be important to determine the genes targeted by this complex.

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DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF:

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1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
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